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Tolerance Following Organophosphate  
Poisoning of Tracheal Muscle

Annual Report

Jerry M. Farley, Ph.D., and Terry M. Dwyer, M.D., Ph.D.

13 December 1984

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
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Department of Pharmacology and Toxicology  
University of Mississippi Medical Center  
Jackson, Mississippi 39216-4505

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from the ratio of  $k_1$  and  $k_{-1}$  was  $26.3 \pm 3.8$  pM, which is close to the value of  $K_D$  calculated from Scatchard plots of binding isotherms. The muscarinic antagonists atropine and scopolamine competitively inhibited [ $^3$ H]QNB binding to the homogenate, with a Hill coefficient of 0.9 and inhibition constant ( $K_i$ ) of 1.8 nM. Muscarinic agonists and the nicotinic antagonist d-tubocurarine inhibited [ $^3$ H]QNB binding in micromolar concentrations. The density of muscarinic receptor binding sites was 10-fold greater in tracheal smooth muscle than in the epithelia ( $0.20 \pm 0.03$  pmole/mg protein). Tolerance to daily diisopropylfluorophosphate (DFP) injection was observed in these animals. The development of tolerance is paralleled by a 10-fold decrease in muscarinic receptor number after 10 days of treatment. Treatment in vitro of muscarinic receptors with DFP had no effect on the receptor density. There was no difference in muscarinic receptor density between young and old swine tracheal smooth muscles.

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## Summary

The tritiated muscarinic cholinergic antagonist quinuclidinyl benzilate ( $[^3\text{H}]\text{QNB}$ ) was used to characterize the muscarinic receptors associated with membranes prepared from swine tracheal smooth muscle. As judged from receptor-binding assays, the membrane homogenate had specific high-affinity receptors for  $[^3\text{H}]\text{QNB}$ . Specific binding was saturable with respect to  $[^3\text{H}]\text{QNB}$  and tissue concentrations and was time- and pH-dependent. The association of  $[^3\text{H}]\text{QNB}$  (180 pM) with the muscarinic receptors reached equilibrium much sooner at 37°C than 25°C (30 min and 2 hrs, respectively). Equilibrium at both temperatures was attained within 5 min at a high concentration of  $[^3\text{H}]\text{QNB}$ , 1800 pM. All other experiments were performed at 37°C. Analysis of binding isotherms yielded an apparent equilibrium dissociation constant ( $K_D$ ) of  $51 \pm 20$  pM and a maximum receptor density ( $B_{\text{max}}$ ) of  $2.17 \pm 0.27$  pmole/mg protein. The Hill coefficient for  $[^3\text{H}]\text{QNB}$  binding was  $1.07 \pm 0.16$ . The association ( $k_1$ ) and dissociation ( $k_{-1}$ ) rate constants were determined to be  $(5.51 \pm 0.16) \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$  and  $(1.41 \pm 0.18) \times 10^{-2} \text{ min}^{-1}$ , respectively.  $K_D$  calculated from the ratio of  $k_1$  and  $k_{-1}$  was  $26.3 \pm 3.8$  pM, which is in reasonable agreement with the value of  $K_D$  calculated from Scatchard plots of binding isotherms. The muscarinic antagonists atropine and scopolamine competitively inhibited  $[^3\text{H}]\text{QNB}$  binding to the homogenate, with a Hill coefficient of 0.9 and inhibition constant ( $K_i$ ) of 1.8 mM. Muscarinic agonists and the nicotinic antagonist d-tubocurarine inhibited  $[^3\text{H}]\text{QNB}$  binding in micromolar concentrations. The density of muscarinic receptor binding sites was 10-fold greater in tracheal smooth muscle than in the epithelia ( $0.20 \pm 0.03$  pmole/mg protein). Tolerance to daily diisopropylfluorophosphonate (DFP) injection was observed in these animals. The development of tolerance is paralleled by a 10-fold decrease in muscarinic receptor number after 10 days of treatment. Treatment *in vitro* of muscarinic receptors with DFP had no effect on the receptor density. There was no difference in muscarinic receptor density between young and old swine tracheal smooth muscles.



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## Foreword

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



## Table of Contents

	Page
Title.....	1
Summary.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	6
Materials and Methods.....	7
Results.....	11
Future Work.....	14
Discussion.....	15
References.....	31
Distribution List.....	34

## Tables and Figures

Table 1: Relative potencies of competitive inhibitors.....	17
Table 2: Distribution of muscarinic receptors.....	18
Table 3: Effect of <u>in vitro</u> DFP on muscarinic receptors.....	19
Table 4: Effect of <u>in vitro</u> DFP on AChE and muscarinic binding.....	20
Table 5: Characteristic symptoms after DFP exposure.....	21
Figure 1: Tissue concentration and [ <sup>3</sup> H]QNB binding.....	22
Figure 2: Effect of pH on [ <sup>3</sup> H]QNB binding.....	23
Figure 3: Time course of [ <sup>3</sup> H]QNB binding.....	24
Figure 4: Kinetics of [ <sup>3</sup> H]QNB binding.....	25
Figure 5: Binding isotherms and Hill coefficient determination.....	26
Figure 6: Displacement of [ <sup>3</sup> H]QNB by muscarinic agonists and antagonists.....	27

	Page
Figure 7: Whole blood acetylcholinesterase after subacute DFP exposure.....	28
Figure 8: Tissue acetylcholinesterase levels after subacute DFP exposure.....	29
Figure 9: Binding isotherms before and after <u>in vitro</u> DFP.....	30

## Introduction

The parasympathetic neuroregulation of smooth muscle contraction/tone is now recognized as an important factor in tracheal function. Bronchoconstriction can be elicited in dogs by stimulation of the cervical branch vagus nerve (1,2,3). This effect appears to be mediated through muscarinic cholinergic receptors, since it can be inhibited by atropine (1). Characterization and localization of these receptors are of importance in understanding how contractile responses are mediated at the receptor level.

The development of radioactive muscarinic ligands with high specific activity and high affinity receptors has facilitated these studies. The tritiated muscarinic antagonist quinuclidinyl benzilate ( $[^3\text{H}]\text{QNB}$ ) has been the most widely used of these ligands for binding assay. With these ligands it has been possible to determine directly the number and pharmacological characteristics of muscarinic cholinergic receptors in several organs, including rat brain (4), guinea pig ileum (5), and heart (6,7,8,9,10). Furthermore, the presence of muscarinic receptors in the tracheal smooth muscles of larger animals such as ferret (11), dog (12), and cow (13) has been reported. However, there is no or low binding in tracheas of small animals such as guinea pig (13) and rat (unpublished data from our laboratory).

There is little information regarding the pharmacological characteristics of the muscarinic cholinergic receptors in swine tracheal smooth muscle. Swine have become widely used animal models for biomedical research (29). The primary utility of swine as animal models is their remarkable anatomic, physiological, nutritional, and metabolic similarities to humans. Their physiological functions such as respiratory, renal, and cardiovascular systems are especially similar to those of humans. Therefore, we have chosen swine to characterize the properties of muscarinic cholinergic receptors in tracheal smooth muscle.

In this study, we used a ligand binding assay to demonstrate and characterize a single population of muscarinic cholinergic receptors in the tracheal smooth muscle. The kinetic properties of the binding and its regional localization were demonstrated. The relationships of pharmacological properties, receptor affinities for agonists and antagonists and density was studied. We compared the distribution of muscarinic cholinergic receptors in various regions of swine trachea and compared these data with those for tracheal smooth muscle from dog (12) and cow (14).

The development of tolerance to the actions of diisopropylfluorophosphonate (DFP) was also studied; evidence for the development of tolerance is discussed.

## Materials and Methods

### Animals and care:

Whole tracheas were obtained from Jackson Packing Co. or from weanling pigs from a local supplier. Pigs were maintained in the animal care facility and fed ad libidum.

### Injection of Animals:

DFP was prepared to a stock concentration of 10 mg/ml in 4°C normal saline. The DFP stock was then frozen in 1 or 2 ml aliquots at -70°C. At the time of injection aliquots were removed from the freezer and rapidly thawed. Weanling swine (6-10 Kg) were injected intramuscularly in the rump with DFP (10 mg/ml stock concentration) dissolved in normal saline. Initially LD<sub>50</sub> was determined by injection of animals with various amounts of DFP (/Kg) and waiting 24 hours to see which animals survived. In general if an animal lived for 2 hours they would survive. The LD<sub>50</sub> was determined to be 5-7 mg/Kg. To induce tolerance development animals were injected with a 2 mg/Kg dose on the first day of injection and 0.5 mg/Kg dose thereafter. Injections were performed between 9:00 and 10:00 A.M. daily. Since only small volumes of saline (>2 ml) were injected we did not do vehicle control. Weanling animals were sacrificed (2 hours or 24 hours after the last DFP injection) by severing the spinal column at the base of the skull utilizing a captive bolt pistol.

### Tissue Preparation:

The brain was removed and sliced choronally at the level of the optic chiasm and the striatum was removed with a wire loop. The duodenal ileum and vas deferens were also removed. All tissues were prepared similarly to that described for trachea below. The trachea and other tissues were rapidly excised and placed in cold oxygenated, balanced salt solution and placed on ice. The isolated smooth muscle was weighed, placed in 10 volumes of 50 mM Na-K phosphate buffer (pH 7.4), and homogenized with a polytron (Kinematica GmbH, Switzerland; setting 10, 2 minutes). The homogenate was centrifuged at 500 x g for 10 minutes; the pellet was discarded and the supernatant was centrifuged at 35,000 x g for 20 minutes to obtain the membrane pellet. The resultant pellet was suspended in 5 volumes of 50 mM Na-K phosphate buffer (pH 7.4), which made the protein concentration of the homogenate 0.3-0.5 mg/ml. This was used for [<sup>3</sup>H]QNB binding assay without further processing. The protein concentration was determined by the method of Lowry et al. (15), using bovine serum albumin as the standard. The tissue was frozen at -20°C until binding assays were performed.

### Muscarinic receptor assay:

The frozen tissue was thawed and homogenized with a polytron (Ultra-Turrax, West Germany; setting 6, 1 minute). The [<sup>3</sup>H]QNB binding assay was performed according to the method of Yamamura and Snyder (5) with little modification. In the standard assay, 0.2 ml of tissue was added to triplicate 10 ml culture test tubes that contained 1.8 ml of 50 mM Na<sub>3</sub>K phosphate buffer (pH 7.4) with varying concentrations of the labeled ligand, [<sup>3</sup>H]QNB (38 ci/ mmole, Amersham Co.). The nonspecific binding assay was performed in the presence of 1 µM atropine (Sigma



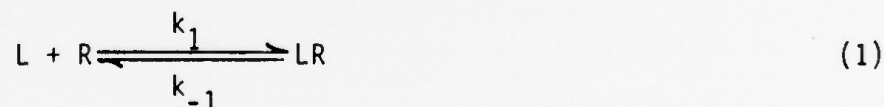
Co.). This mixture was incubated at 37°C for 45 minutes. The reaction was terminated by rapid filtration under vacuum through Whatman GF/B fiber filters. The filters had been previously soaked in 0.05% polyethyleneimine (16) for 5 minutes and rinsed four times with 50 mM Na-K phosphate buffer. After the reaction mixture was filtered, the filters were washed rapidly four times with 5 ml of chilled 50 mM Na-K phosphate buffer (5°C). Each filter was placed in a vial containing 5 ml of PCS solution (Amersham Co.) and the radioactivity was determined by liquid scintillation counting (Tracor Analytic Mark III). All radioactivity values were corrected for specific activity, quenching and counting efficiency. The amount of specific binding was calculated as the total binding minus the binding in the presence of 1  $\mu$ M atropine. The equilibrium displacement of [ $^3$ H]QNB was performed to permit estimation of the potency of various muscarinic antagonists to inhibit QNB binding. A single concentration of [ $^3$ H]QNB (100-180 pM) was equilibrated with tissue homogenate in the presence of various concentrations of cold competing ligand. The ligands used were the inhibitors d-tubocurarine, atropine, scopolamine and the agonists pilocarpine, carbachol and acetylcholine. In order to eliminate hydrolysis of acetylcholine, due to the presence of pseudo- and acetylcholinesterase in the homogenate, physostigmine (1  $\mu$ M) was added.

#### Kinetic assays:

Kinetic studies were carried out using the same filtration method described by Murals et al. (12). To determine the association rate constant and time course. In this assay, 9 ml of membrane homogenate was added to a 250 ml flask containing 81 ml of 50 mM Na-K phosphate buffer with [ $^3$ H]QNB (100 pM). Nonspecific binding was determined in the presence of 1  $\mu$ M atropine. The specific binding of [ $^3$ H]QNB was determined after various incubation times at 37°C. In another flask containing the same solution, atropine at a final concentration of 1  $\mu$ M was added after 45 minutes of incubation. At timed intervals, 2-ml aliquots were removed and filtered. The dissociation rate constant was determined in a similar way. The tissue was equilibrated with 100 pM [ $^3$ H]QNB at 37°C for 45 minutes. After equilibration, atropine was added to the mixture at a final concentration of 1  $\mu$ M and then at timed intervals, 2 ml samples were removed and filtered. All the assays were performed in triplicate.

#### Analysis of binding data:

For the analysis of binding data, it was assumed that the reaction of the muscarinic cholinergic receptor (R) with muscarinic agonists, antagonists, or the radioligand [ $^3$ H]QNB (L) obeyed the law of mass action, and thus:



where LR is the ligand-receptor complex formed. At equilibrium (e), the dissociation constant ( $K_D$ ) is defined as:

$$K_D = (R)_e \cdot (L)_e / (LR)_e \quad (2)$$



measured within 4 hr of sacrifice. AChE was assayed by the colorimetric method of Ellman et al. (21), as modified by Benke et al. (22), using S-acetylthiocholine iodide as a substrate. The reaction involves the formation of the colored 5-thio-2-nitrobenzoate anion by the reaction of released thiocholine with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). The butyrylcholinesterase assay was similar to that for AChE except that butyrylthiocholine iodide was used as a substrate. Esterases were assayed by adding an aliquot of tissue homogenate (50-400  $\mu$ l) or 5-20  $\mu$ l of whole blood, 20  $\mu$ l of 1.0 M S-acetylthiocholine iodide and 100  $\mu$ l of 0.1 M DTNB to a cuvette containing 2.5 ml of 0.1 M phosphate buffer (pH 8.0). The absorbance was scanned for at least 10 min using a spectrophotometer (Gilford Instrument Co.) at 412 nm. The initial absorbance, as well as a reagent blank absorbance, were subtracted from the final reading. The activity of AChE was expressed as  $\mu$ mole S-acetylthiocholine (or butyrylthiocholine) iodide hydrolyzed/min/mg protein for tissue or /min/ml of whole blood. The protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

## Results

### Effects of protein concentration and pH on [ $^3$ H]QNB binding:

Specific [ $^3$ H]QNB binding increased linearly with increasing tissue concentrations from 0.01 to 0.7 mg protein in the [ $^3$ H]QNB concentration range from 20 to 400 pM (Fig. 1). The data shown in Fig. 1 were obtained in the presence of 118 pM [ $^3$ H]QNB. Specific binding remained constant over a pH range of 7.0 to 8.0 but displayed a small maximum at pH 7.4 (Fig. 2). The binding assays for the remainder of these studies were conducted with less than 0.7 mg of protein at pH 7.4.

### Determination of equilibrium time:

Binding of [ $^3$ H]QNB to a crude membrane preparation from swine tracheal smooth muscle was dependent on time, temperature and pH. To determine the time required to reach equilibrium at both 37°C and 25°C, the time courses of [ $^3$ H]QNB binding to tracheal smooth muscle membrane were examined. Fig. 3 shows that the binding of [ $^3$ H]QNB to the homogenate of swine tracheal smooth muscle reached equilibrium much sooner at 37°C than at 25°C at a [ $^3$ H]QNB concentration of 180 pM. The time to reach equilibrium was about 35 minutes at 37°C and 120 minutes at 25°C. Half-maximal binding occurred within 3-4 minutes at 37°C and 30 minutes at 25°C. With a higher concentration of [ $^3$ H]QNB (1000 pM), equilibrium was reached within 5 minutes at both temperatures (data not shown). Nonspecific binding was not saturable and increased linearly with increasing [ $^3$ H]QNB concentration. The equilibrium time point determined at 37°C was therefore chosen for further experiment throughout this study.

### Kinetic constants for [ $^3$ H]QNB binding:

The specific [ $^3$ H]QNB binding to swine tracheal smooth muscle membrane reached equilibrium at 37°C by 35 minutes (Fig. 4A). Kinetic data from Fig. 4A were used to calculate a second order forward rate constant,  $K_1$ , of  $(5.51 \pm 0.16) \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$  (Fig. 4B). Dissociation of [ $^3$ H]QNB binding was slow with a half-life of 50 minutes (Fig. 4A). A first order plot of the data of Fig. 4A shows the dissociation of [ $^3$ H]QNB to be monophasic (Fig. 4C). The dissociation rate constant ( $K_{-1}$ ) calculated from the first order plot was  $(1.41 \pm 0.18) \times 10^{-2} \text{ min}^{-1}$ . Atropine (1  $\mu\text{M}$ ) was used as the quenching agent in the determination of the dissociation rate constant. No other antagonist was used for this purpose. A  $K_D$  value of  $26.3 \pm 3.8 \text{ pM}$ , calculated from the ratio of the rate constants  $K_{-1}/K_1$ , was not significantly different from the average  $K_D$  determined by Scatchard analysis of saturation isotherms (next subsection).

### Saturability of [ $^3$ H]QNB binding:

The saturability of [ $^3$ H]QNB binding to swine tracheal smooth muscle membrane was measured by incubating at 37°C for 45 minutes with varying concentrations of [ $^3$ H]QNB from 20 to 1500 pM (Fig. 5A). The saturation isotherm is a rectangular hyperbola, suggesting that swine tracheal smooth muscle membrane contains a single population of saturable high affinity muscarinic cholinergic receptors. There was no evidence of the existence of a second population of saturable binding sites of lower affinity even when the [ $^3$ H]QNB

concentration was raised to 2000 pM. Nonspecific binding, on the other hand, increased linearly with increasing [ $^3\text{H}$ ]QNB concentration up to 2000 pM. Scatchard plot analysis (17) of specific bound [ $^3\text{H}$ ]QNB (Fig. 5A, inset) gave an apparent dissociation constant ( $K_D$ ) of  $51 \pm 20$  pM ( $n=9$ ) and a maximal number of binding sites ( $B_{\text{max}}$ ) of  $2.1 \pm 0.3$  pmole/mg protein. The correlation coefficients for the Scatchard plots ranged from 0.87 and 0.99 ( $n=9$ ). A plot of binding data according to the Hill equation (20) gave a straight line with a Hill coefficient  $n_H = 1.07 \pm 0.16$ , suggesting the absence of cooperative binding interaction (Fig. 5B).

#### Pharmacological specificity of [ $^3\text{H}$ ]QNB binding:

If specific [ $^3\text{H}$ ]QNB binding reflects an interaction with the muscarinic cholinergic receptors, muscarinic drugs should have significant affinity for the same binding sites. The pharmacological specificity of binding was measured by studying the effects of a variety of muscarinic antagonists, agonists and nonmuscarinic drugs on [ $^3\text{H}$ ]QNB binding (Fig. 6). All the drugs displaced [ $^3\text{H}$ ]QNB from muscarinic receptors in a dose-dependent manner. Hill coefficients and inhibition constants ( $K_i$ ) for each competitive interaction were calculated from competition data (Table 1). Half-maximal inhibition ( $\text{IC}_{50}$ ) of [ $^3\text{H}$ ]QNB binding by the muscarinic antagonists atropine and scopolamine was obtained at nanomolar concentrations. The  $K_i$  was 1.8 nM and the Hill coefficient was 0.9. The nicotinic antagonist d-tubocurarine also inhibited [ $^3\text{H}$ ]QNB binding at micromolar concentrations. The cholinergic agonists such as acetylcholine, carbachol and pilocarpine were much less effective as inhibitors, exhibiting  $K_i$ 's in the micromolar range. The Hill coefficients were in the 0.5-0.75 range.

#### Distribution of muscarinic cholinergic receptors in swine trachea:

Preliminary experiments were performed to estimate the muscarinic cholinergic receptor density in homogenates from the epithelia and smooth muscle of swine trachea. When membranes for these regions were prepared, specific binding of [ $^3\text{H}$ ]QNB (115 pM) was higher in the smooth muscle homogenate. The maximum receptor densities for smooth muscle and epithelia were  $2.17 \pm 0.27$  and  $0.20 \pm 0.03$  pmole/mg protein, respectively (Table 2). Therefore, all the experiments in this study were performed by using swine tracheal smooth muscle homogenates which were carefully cleaned of epithelial tissue.

#### Tolerance development to DFP action:

Pigs were injected daily (at 9:00-10:00 AM) with DFP (2 mg/kg loading dose, 0.5 mg/kg daily thereafter). The pigs initially demonstrated the classical signs and symptoms of organophosphate poisoning: diarrhea, vomiting, salivation, muscle tremors and muscle weakness (Table 5). However, after several days of treatment, these signs and symptoms had either disappeared or been greatly reduced. This recovery occurred even though the levels of acetylcholinesterase activity in the tissues and blood were drastically reduced (Fig. 7 and 8). We have found that a significant recovery of butyrylcholinesterase occurs in whole blood within 24 hours after the last DFP injection. This recovery evidently represents the formation of new enzyme and may help to explain the need for multiple injections of DFP to bring about tolerance development. Newly synthesized circulating levels of

butyrylcholinesterase could hydrolyze acetylcholine thus removing the primary stimulus for down-regulation of muscarinic receptors. Specific binding of the muscarinic antagonist [ $^3$ H]QNB in tracheal smooth muscle was greatly reduced after several days of DFP treatment. For example, in control animals the maximum specific binding in tracheal smooth muscle was approximately 2 pmoles/mg. After 10 days of DFP treatment, the maximum specific binding in tracheal muscle was reduced by about 10-fold to 0.2 pmole/mg protein. The affinity was not changed. More recent experiments have illustrated that the decrease in receptor number observed at day 10 is reached by day 7 of injection. A decline in receptor occurs from day 0 to day 7. Thus one possible mechanism for the development of tolerance to organophosphates is a decrease in muscarinic receptor number, which results in a decreased sensitivity of the tissue to acetylcholine. The effects on receptor number cannot be explained by a direct action of DFP on the receptors. This is illustrated in Fig. 9 and Table 3. DFP, even at a concentration of 1 mM, had no effect on either  $B_{max}$  or  $K_D$ . Thus the decrease in receptor number was due to a down-regulation of the receptors. In a preliminary observation, after 1 and up to about 3 days of DFP treatment (i.e., 0.5 mg/kg/day), the affinity of the muscarinic receptor for atropine decreased. We observed this only indirectly as a curvature in the nonspecific portion of the binding isotherms. We are now performing experiments to determine whether this change is real or is some artifact of the conditions under which the binding study was performed. Table 4 demonstrates that tracheal epithelium also has muscarinic receptors. These are at much lower density than those in muscle. Therefore, it is important to remove the epithelium if a good estimate of tracheal muscle muscarinic receptor density is to be made.



## Future Work

### Nerve agents:

We are now ready to utilize the agents soman, sarin and tabun. We will be formally requesting the shipment of the agents soon.

### Muscle contraction:

We have set up the muscle contraction apparatus and will be performing experiments analogous to those performed in the binding study. We will be measuring dose-response relationships for acetylcholine, carbachol and histamine in control and tolerant animals.

### Electrophysiology:

The electrophysiological portions of the experiments were to be performed late in Year 2 and Year 3. However, we have begun to work out the techniques for enzymatic dispersion of smooth muscle cells. We are going to attempt to grow these cells in culture.



## Discussion

The contraction of tracheal and bronchial smooth muscle is predominantly under parasympathetic control. Following administration of atropine, tracheal and bronchial smooth muscles relax (1). In this study, we have characterized the muscarinic cholinergic receptors of swine tracheal smooth muscle. Our findings demonstrate the existence of a single population of muscarinic cholinergic receptors in this tissue.

[<sup>3</sup>H]QNB binding was used to identify and characterize muscarinic cholinergic receptors on tracheal smooth muscle. The tracheal preparation consisted mainly of smooth muscle and was found to have specific, saturable, high affinity muscarinic cholinergic binding sites. Scatchard analysis of binding data indicated a  $K_D$  of  $51 \pm 20$  pM and  $B_{max}$  of  $2.1 \pm 0.3$  pmol/mg protein. The binding characteristics were similar to those of the muscarinic cholinergic receptors described for the rat brain (4,5,25), heart (6,7,8,9,10), ileum (5), smooth muscles of the lower esophageal sphincter (23), pylorus (24), and dog trachea (12). Thus, the muscarinic cholinergic receptors consist from swine trachea of a single population of saturable binding sites, have greater affinity for muscarinic antagonists than for agonists, and display appropriate pharmacological specificity. The rostral to caudal gradient of muscarinic receptors in tracheal smooth muscle, known to exist in other animals, was not examined in this study.

Williams and Lefkowitz (26) have emphasized that the interaction of ligand with receptor occurs rapidly. The half-time for association of [<sup>3</sup>H]QNB with the homogenate of swine tracheal smooth muscle was estimated to be 3-4 minutes, determined at 37°C. The equilibrium  $K_D$  of  $26.3 \pm 3.8$  pM as determined by kinetic experiments was not significantly different from the  $K_D$  determined from our saturation experiments ( $51 \pm 20$  pM). This indicates that the assumption of a simple bimolecular reaction is probably valid. Dissociation was very slow, with a half-life of 50 minutes at 37°C.

In comparison, the maximum density of muscarinic cholinergic receptors in the swine tracheal smooth muscle ( $2.17 \pm 0.27$  pmol/mg protein) agreed well with the data ( $2.805 \pm 0.309$  pmoles/mg) from Cheng and Townley (14) and was five times the number ( $0.410 \pm 0.034$  pmol/mg protein) obtained from dog whole tracheal smooth muscle (12).

All the muscarinic drugs displaced [<sup>3</sup>H]QNB binding in a dose-dependent manner. In general, muscarinic antagonists had an apparent  $K_i$  in the nanomolar range, whereas agonists had a  $K_s$  in the micromolar range. The Hill coefficients from [<sup>3</sup>H]QNB binding data were  $1.07 \pm 0.16$  and 0.9 for muscarinic antagonists displacing [<sup>3</sup>H]QNB binding, as expected for an interaction between two muscarinic antagonists competing for a homogenous class of non-interacting binding sites on the muscle. The muscarinic agonists, however, gave Hill coefficients of 0.5-0.75. Birdsall et al. (27) and more recently Schimerlik and Searles (28) and Halvorsen and Nathanson (29) have suggested that Hill coefficients for agonists that are significantly less than 1.0 arise from heterogeneity in the affinity of the muscarinic cholinergic receptor population for these compounds.

In conclusion, our experiments demonstrate that ligand binding assays are an effective means of directly measuring parameters of receptor occupancy in homogenates of isolated swine tracheal smooth muscle. The swine tracheal smooth muscle exhibits high-affinity, specific [<sup>3</sup>H]QNB binding. This binding has been characterized by displacement analysis to have properties consistent with muscarinic cholinergic receptors.

The organophosphate DFP causes signs and symptoms in the swine consistent with muscarinic overstimulation. These symptoms decrease with time until only some tremor and muscle weakness is observed. The decrease in signs and symptoms is paralleled in tracheal muscle by a 10-fold decrease in the density of receptors. This decrease in receptor number no doubt plays a role in the decrease in signs and symptoms observed. This decrease is not due to any direct actions of DFP, since even at 1 mM, the organophosphate did not alter receptor binding in vitro.

TABLE 1. Relative potencies of drugs as competitive inhibitors of [<sup>3</sup>H]QNB binding in tracheal smooth muscle homogenate.<sup>a</sup>

Drug	IC <sub>50</sub> (M) <sup>b</sup>	K <sub>i</sub> (M)	Hill Coefficient
Atropine	5.03X10 <sup>-9</sup>	1.80X10 <sup>-9</sup>	0.91
Scopolamine	4.70X10 <sup>-9</sup>	1.74X10 <sup>-9</sup>	0.90
Acetylcholine <sup>c</sup>	6.24X10 <sup>-6</sup>	2.24X10 <sup>-6</sup>	0.51
Carbachol	2.17X10 <sup>-6</sup>	0.78X10 <sup>-6</sup>	0.60
Pilocarpine	1.47X10 <sup>-5</sup>	0.53X10 <sup>-5</sup>	0.75
Tubocurarine	3.76X10 <sup>-5</sup>	1.79X10 <sup>-5</sup>	0.59

<sup>a</sup>Membrane homogenates (0.054 mg of protein) were incubated with 100 pM [<sup>3</sup>H]QNB for 45 minutes at 37°C.

<sup>b</sup>Molar concentration of drug which inhibited 50% of the specific [<sup>3</sup>H]QNB binding. The IC<sub>50</sub> values were determined from a log-probit plot of data like that in Fig. 6. Values are the means of two experiments, each point done in triplicate. Range for all of the values is less than 20%.

<sup>c</sup>Incubations also contained 1 μM physostigmine to prevent hydrolysis of acetylcholine.

TABLE 2. Comparison of muscarinic receptor densities in tracheal tissue homogenates.<sup>a</sup>

Tissue	B <sub>max</sub> (pmol/mg)	K <sub>D</sub> (pM)	n <sup>b</sup>
Smooth muscle	2.17 ± 0.27	51 ± 20	9
Smooth muscle + epithelium	1.27 ± 0.38	51 ± 30	3
Epithelium	0.20 ± 0.03	67 ± 20	3

<sup>a</sup>Data shown are means ± S.D.

<sup>b</sup>Experiment number.

TABLE 3. Effect of DFP on [ $^3\text{H}$ ]-QNB binding in vitro.<sup>a</sup>

Treatment	$B_{\text{max}}$ (pmol/mg)	$K_D$ (pM)
Control	$1.113 \pm 0.029^b$	$131 \pm 41$
1 mM DFP	$0.937 \pm 0.165$	$93 \pm 33$
500 $\mu\text{M}$ DFP	$1.166 \pm 0.330$	$106 \pm 29$
100 $\mu\text{M}$ DFP	$1.470 \pm 0.366$	$101 \pm 33$
10 $\mu\text{M}$ DFP	$1.259 \pm 0.295$	$98 \pm 8$

<sup>a</sup>Intact trachea were treated with various concentrations of DFP at 37°C for 20 minutes. Preparation of homogenate and receptor binding assay followed our standard methods. The data were expressed as means  $\pm$  S.D.

<sup>b</sup>Mean  $\pm$  standard error of the mean. 3-5 experiments.



TABLE 4. Comparison of receptor changes and inhibition of cholinesterases after in vitro treatment with 1 mM DFP.

Tissue	Treatment	$B_{max}$	$K_D$	AChE <sup>c</sup>	BuChE <sup>d</sup>
		(pmol/mg)	(pM)	( $\mu$ mol/min/mg)	
SM <sup>a</sup> + E <sup>b</sup>	Control	1.646	86	0.00721	0.000586
	1 mM DFP	1.552	91	0	0
SM	Control	2.360	96	0.00477	0.000530
	1 mM DFP	2.658	100	0	0
E	Control	0.202	83	0.00192	0.000431
	1 mM DFP	0.191	140	0	0

mean of 3-5 determinations

<sup>a</sup>Tracheal smooth muscle

<sup>b</sup>Tracheal epithelium

<sup>c</sup>Acetylcholinesterase

<sup>d</sup>Butyrylcholinesterase

TABLE 5. Characteristics of DFP exposure<sup>a</sup>.

	DFP
Speed of action	++ <sup>b</sup>
Diarrhea, vomiting	++++
Tremor	+++
Weakness	++
Shock	+
Salivation	++++
Bronchoconstriction	++

<sup>a</sup>These symptoms occur within 4 hours after an injection of 2-3 mg/kg.

<sup>b</sup>The rating system is based on + being lowest and ++++ being highest.

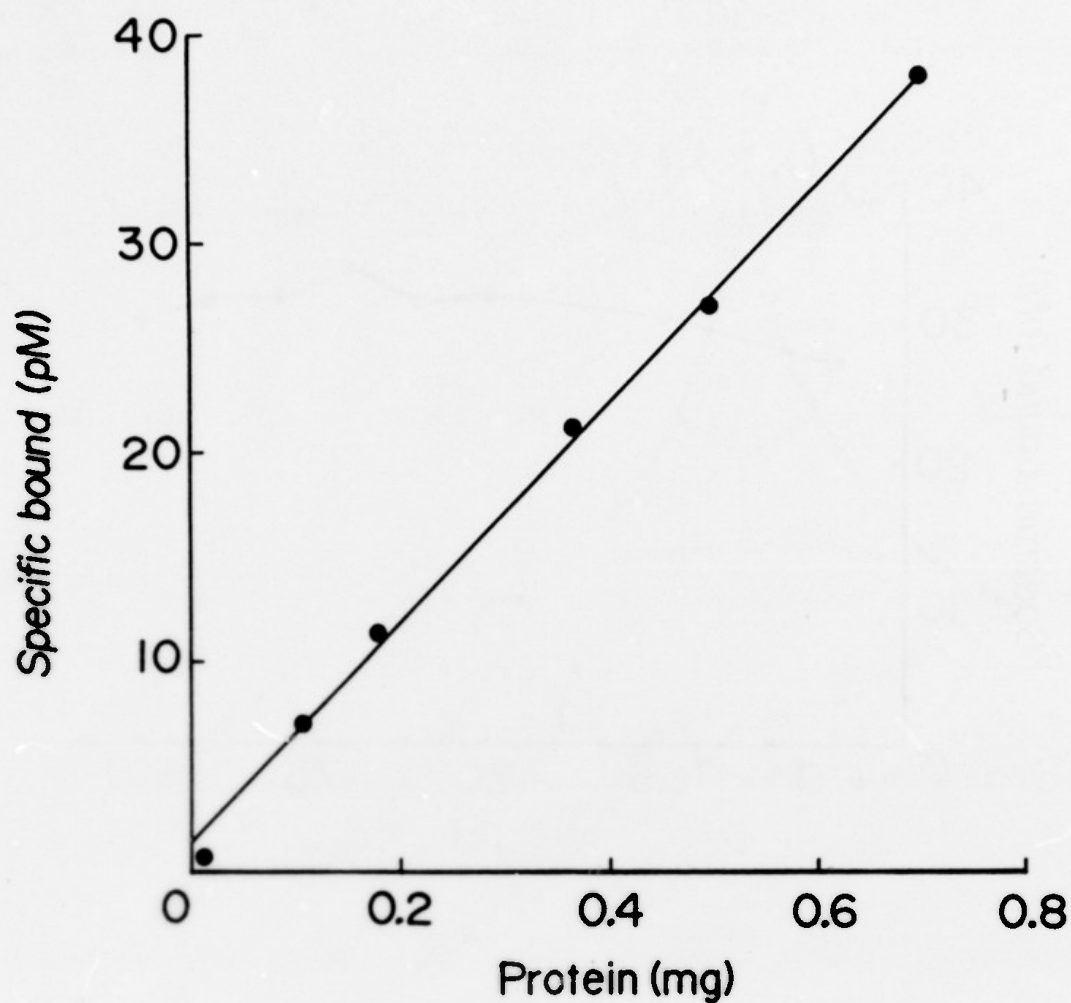


FIGURE 1

Specific binding of [ $^3\text{H}$ ]QNB as a function of tissue concentration. Various amounts of smooth muscle membrane were incubated at  $37^\circ\text{C}$  for 45 minutes in 2.0 ml of Na-K phosphate buffer (pH 7.4) with 118 pM [ $^3\text{H}$ ]QNB. The relationship between protein concentration and binding was linear in this range. Data presented from a single experiment.

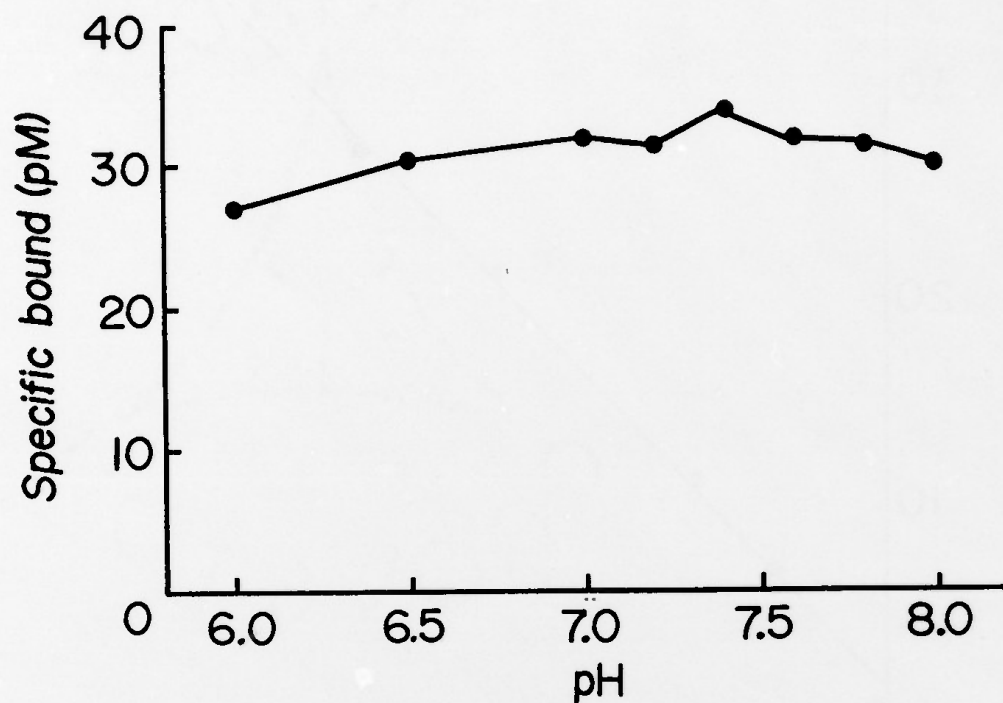


FIGURE 2

Specific binding of  $[^3\text{H}]\text{QNB}$  as a function of pH. Tracheal smooth muscle homogenate was added to 100 pM  $[^3\text{H}]\text{QNB}$  in 2.0 ml of Na-K phosphate buffer (pH 6-8) at 37°C for 45 minutes. This pH range does not greatly affect specific binding. All remaining binding assays were performed at pH 7.4. Data presented for one experiment.

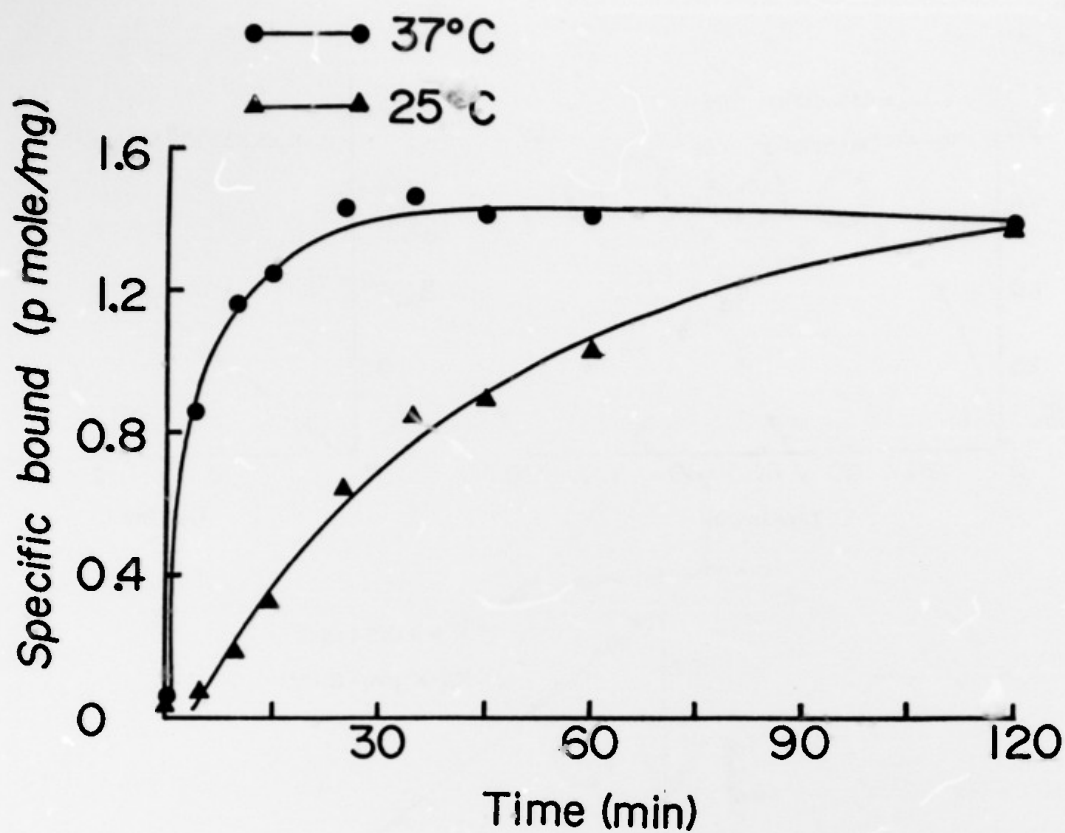


FIGURE 3

Time courses of  $[^3\text{H}]\text{QNB}$  binding.  $[^3\text{H}]\text{QNB}$  (180 pM) binding was measured at 37°C and 25°C with membrane prepared from tracheal smooth muscle (0.044 mg/ml). Data shown are the averages of specifically bound  $[^3\text{H}]\text{QNB}$  (p mole/mg) from triplicate determinations, all performed with one preparation of homogenate.



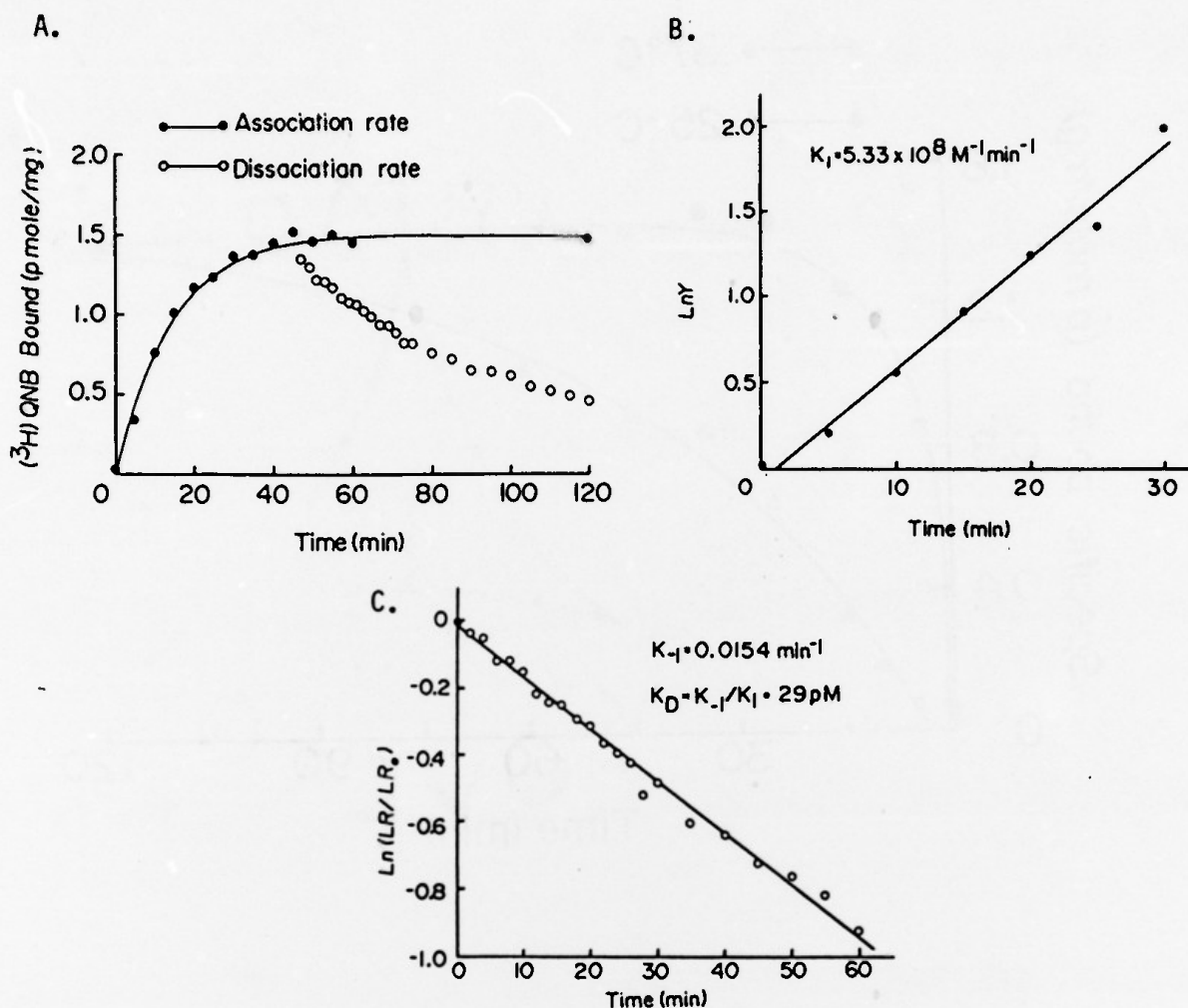


FIGURE 4

A: Time courses for specific binding and dissociation of  $[^3\text{H}]\text{QNB}$  on membrane prepared from tracheal smooth muscle. The data shown are the average from one representative experiment done in triplicate.

B: Kinetic plot for specific  $[^3\text{H}]\text{QNB}$  binding. Data in Fig. 4A from 0 to 30 minutes were used to determine the association rate constant ( $k_1$ ). Data are presented as a second order replot of the data in Fig. 4A according to equation 3.

$$Y = \frac{\text{LR}_e [\text{L}_T - (\text{LR} \cdot \text{LR}_e / \text{R}_T)]}{\text{L}_T (\text{LR}_e - \text{LR})}$$

Note:  $\text{L}_T = [^3\text{H}]\text{QNB}$  concentration;  $\text{R}_T = \text{B}_{\text{max}}$ ;  $\text{LR}$ , specific binding at time  $t$ ;  $\text{LR}_e$ , specific binding at equilibrium.

C: Kinetic plot for determining the dissociation rate constant ( $k_{-1}$ ) of  $[^3\text{H}]\text{QNB}$  binding. The data of Figure 4A were replotted in a first order mode according to equation 4.  $\text{LR}$ =specific binding at time  $T$ .  $\text{LR}_0$ =specific binding at time 0. These data are from single representative experiments.

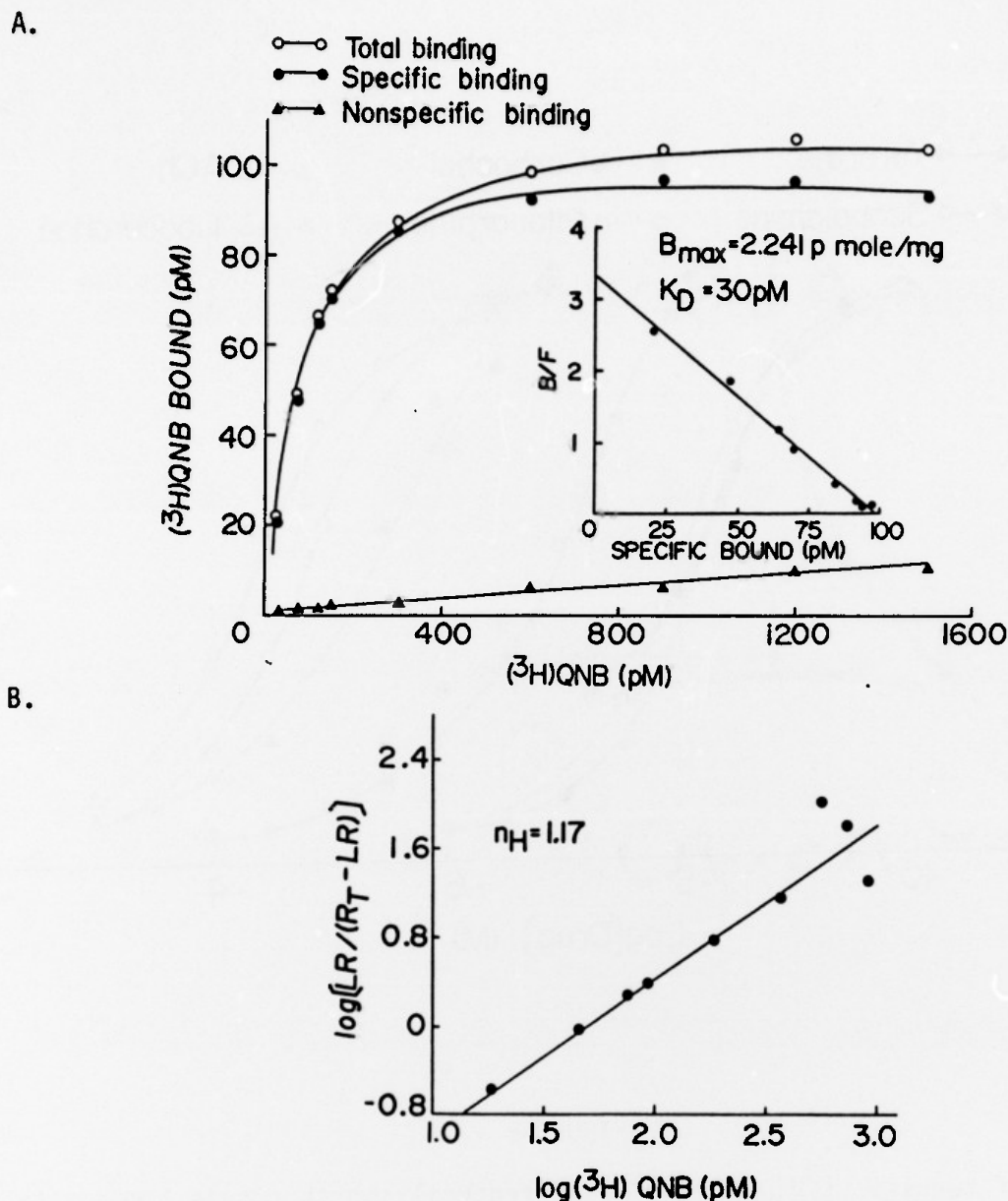


FIGURE 5

A: Determination of the equilibrium dissociation constant for  $[^3\text{H}]\text{QNB}$  binding. Data were expressed as the average values of triplicate determinations in one representative experiment. Inset: Scatchard plot of saturation binding isotherm. The X intercept of a least squares fit to the Scatchard plot is a measure of  $B_{\text{max}}$  and the slope is  $-1/K_D$ . These data are from a single experiment. The mean  $\pm$  S.D. for the equilibrium dissociation constant ( $K_D$ ) from experiments was  $51 \pm 20 \text{ pM}$ ; maximal number of binding sites ( $B_{\text{max}}$ ) and the Hill coefficient were,  $2.17 \pm 0.27 \text{ pmoles/mg protein}$  and  $n_H = 1.07 \pm 0.16$ , respectively ( $n=9$ ).

B: Hill plot of specific  $[^3\text{H}]\text{QNB}$  binding according to the Hill equation with a Hill coefficient of 1.17. These data are from a single representative experiment.

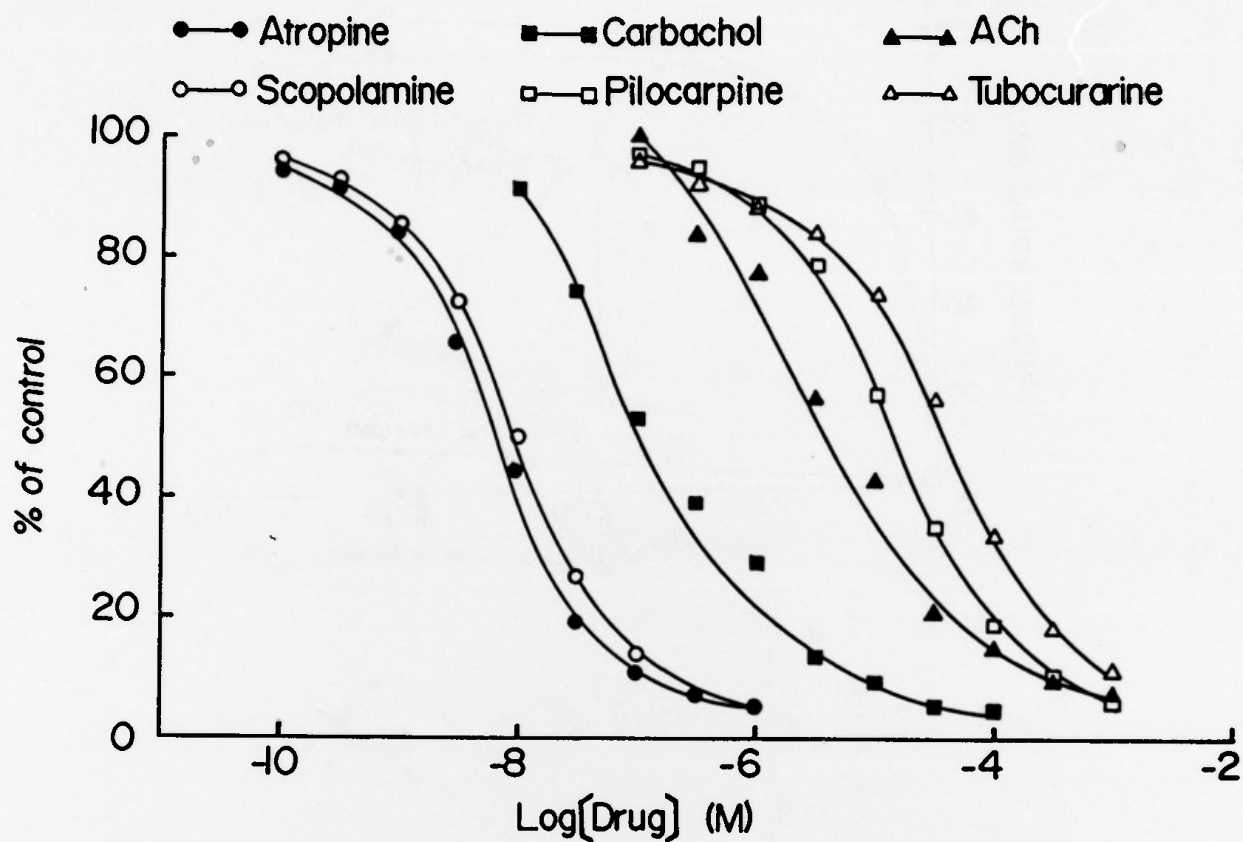


FIGURE 6

Inhibition of specific  $[^3\text{H}]\text{QNB}$  binding to tracheal smooth muscle homogenate by various drugs.  $[^3\text{H}]\text{QNB}$  concentration was 100 pM. Tissue concentration was 0.054 mg/ml. The  $K_i$  value for each drug, calculated from equation 10, is shown in Table 1. The lines are drawn by eye. The data are from representative experiments.

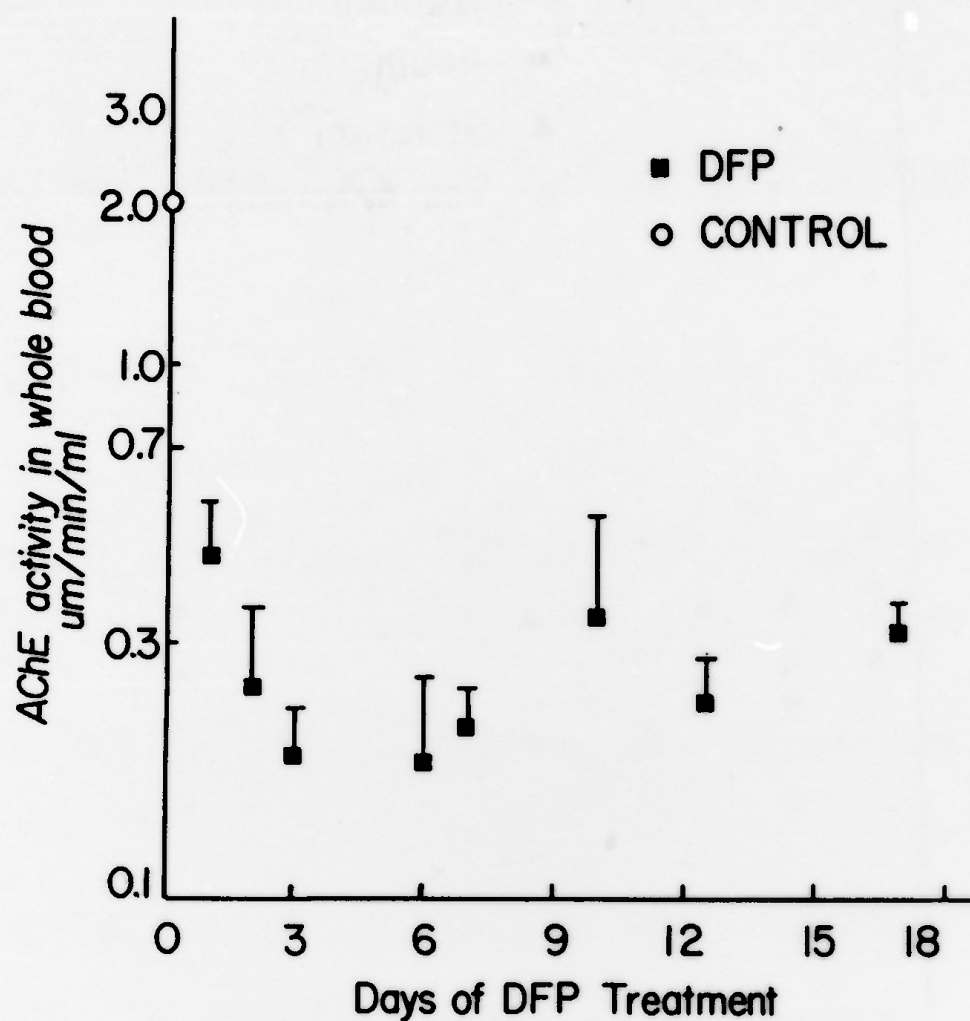


FIGURE 7

The decrease in whole blood AChE activity with daily DFP treatment. Levels dropped after 1 day of treatment to about 25% of control. The means  $\pm$  S.D. are shown for 3-5 animals.



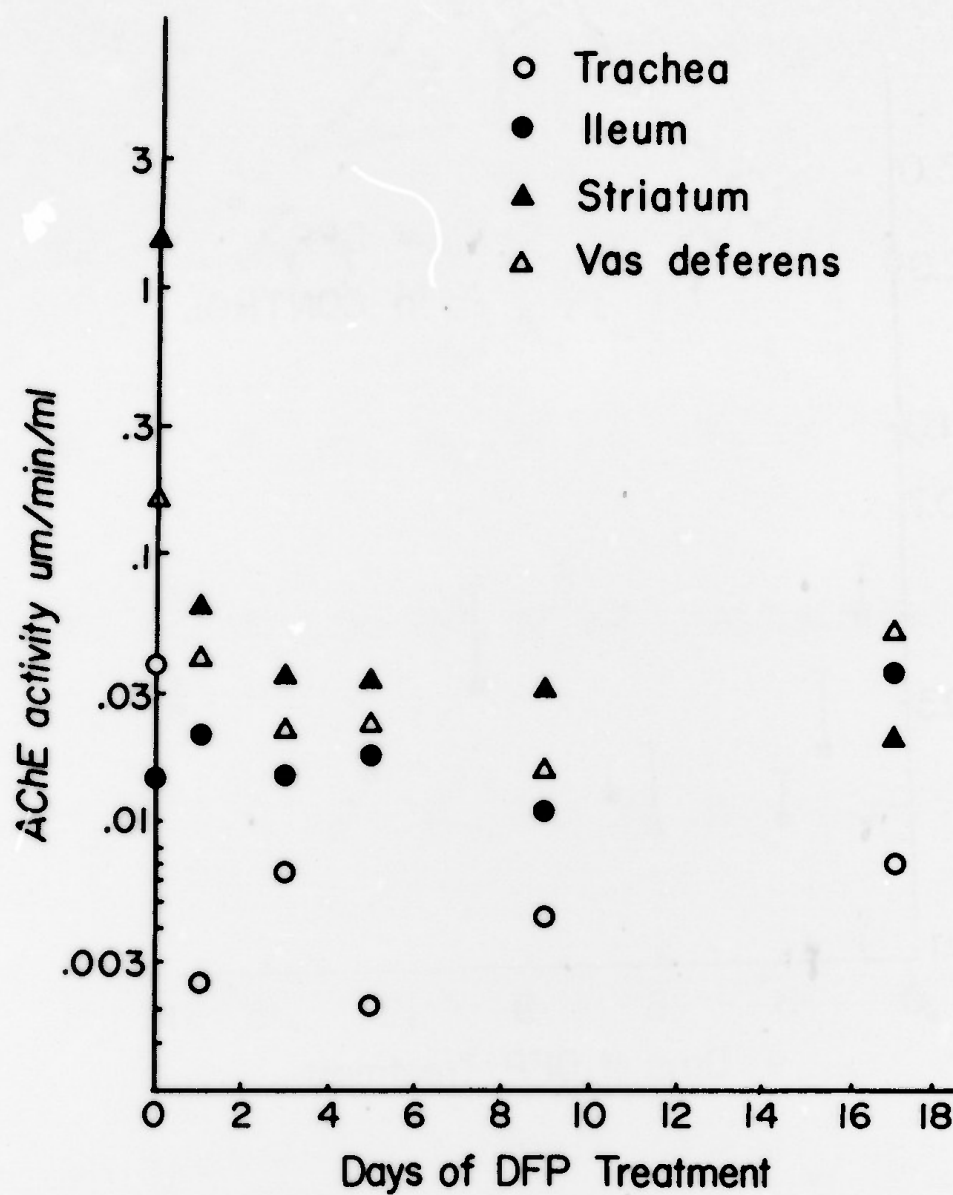
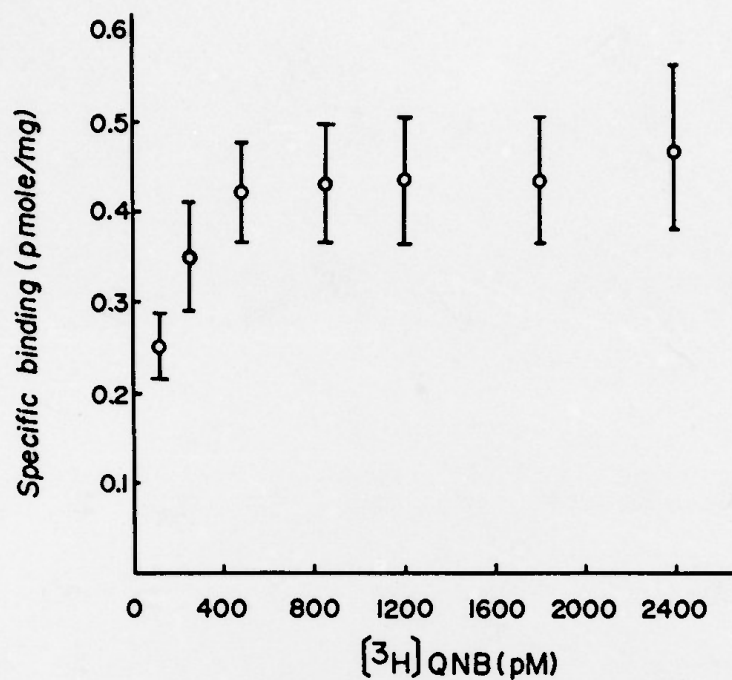


FIGURE 8

Effect of daily treatment with DFP on AChE levels in tissue homogenates. AChE levels decreased to about 16% of control, and remained at this level for the duration of the injection protocol. The mean data are shown for 3-5 animals. Standard deviations are not shown for clarity.



A.



B.

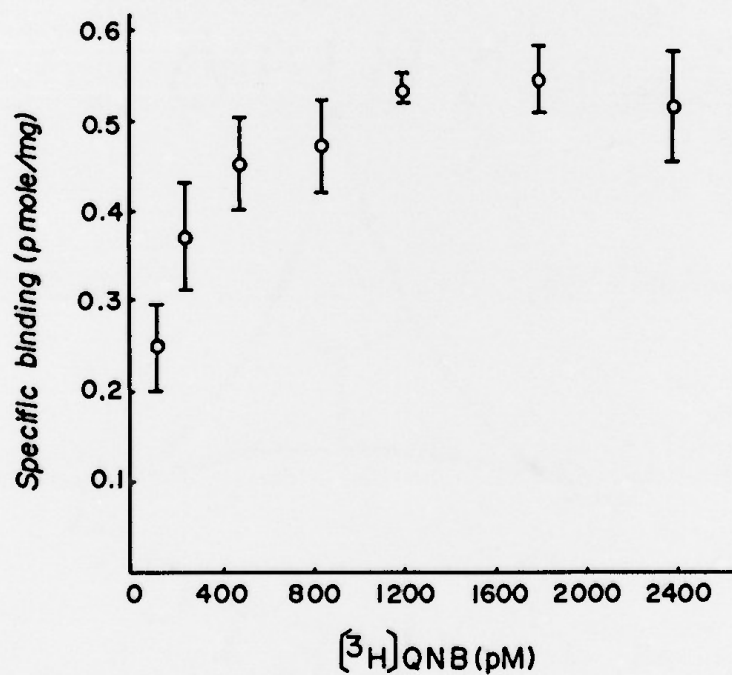


FIGURE 9

A: Binding isotherm in control tracheal muscle.  
 B: Binding isotherm from tracheal muscle treated *in vitro* with 1 mM DFP. No effect on  $K_D$  or  $B_{max}$  occurs. The points are the means  $\pm$  S.D. for 3-5 experiments.

## References

1. Colebatch, H. J. H., and D. F. J. Halmagyi. Effect of vagotomy and vagal stimulation on lung mechanics and circulation. *J. Appl. Physiol.* 18: 881-887, 1963.
2. Karczewski, W., and J. G. Widdicombe. The effect of vagotomy, vagal cooling and efferent vagal stimulation on breathing and lung mechanics of rabbits. *J. Physiol. London.* 201: 259-270, 1969.
3. Olsen, C. R., H. J. J. Colebatch, P. E. Mebel, J. A. Nadel, and N. C. Staub. Motor control of pulmonary airways studied by nerve stimulation. *J. Appl. Physiol.* 20: 202-208, 1965.
4. Yamamura, H. I., and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. U.S.A.* 71: 1725-1729, 1974.
5. Yamamura, H. I., and S. H. Snyder. Muscarinic cholinergic receptor binding in longitudinal muscle of guinea pig ileum. *Mol. Pharmacol.* 10: 861-867, 1974.
6. Fields, J. Z., W. R. Roeske, E. Morkin, and H. I. Yamamura. Cardiac muscarinic cholinergic receptors. *J. Biol. Chem.* 253: 3251-3258, 1978.
7. Wei, J. W., and P. V. Sulakke. Properties of the muscarinic cholinergic receptor in rat atrium. *Naunyn - Schmiedeberg's. Arch. Pharmacol.* 309: 259-269, 1979.
8. Sharma, V. K., and S. P. Banerjee. Muscarinic cholinergic receptors in rat heart. *J. Biol. Chem.* 252: 7444-7446, 1977.
9. Hartzell, H. C. Distribution of muscarinic acetylcholine receptors and presynaptic nerve terminals in amphibian heart. *J. Cell Biol.* 86: 6-20, 1981.
10. Hosey, M. M., and J. Z. Fields. Quantitative and qualitative differences in muscarinic cholinergic receptors in embryonic and newborn chick hearts. *J. Biol. Chem.* 256: 6395-6399, 1981.
11. Basbaum, C. B., M. A. Grillo, and J. H. Widdicombe. Muscarinic receptors: Evidence for a nonuniform distribution in tracheal smooth muscle and exocrine gland. *J. Neurosci.* 4: 508-520, 1984.
12. Murals, C., J. A. Nadel, and J. M. Roberts. The muscarinic receptors of airway smooth muscle: their characterization in vitro. *J. Appl. Physiol.* 52: 1084-1091, 1982.
13. Morris, P. L., and J. S. Douglas. Cholinergic receptors in mammalian lung. *Fed. Proc.* 42: 905, (abstract), 1983.

14. Cheng, J. B., and R. G. Townley. Comparison of muscarinic and beta adrenergic receptors between bovine peripheral lung and tracheal smooth muscles: A striking difference in the receptor concentration. *Life Sci.* 30: 2079-2086, 1982.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* 193: 265-275, 1951.
16. Costa, L. G., and S. D. Murphy. [<sup>3</sup>H] Nicotine binding in rat brain: alteration after chronic acetylcholinesterase inhibition. *J. Pharmacol. Exp. Ther.* 226: 392-397, 1983.
17. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* 51: 660-672, 1949.
18. Weiland, G. A., and P. B. Molinoff. Quantitative analysis of drug-receptor interactions: I. Determination of kinetic and equilibrium properties. *Life Sci.* 29: 313-330, 1981.
19. Cheng, Y., and W. H. Prusoff. Relationship between the inhibition constant ( $K_i$ ) and the concentration of inhibitor which causes 50 per cent inhibition ( $I_{50}$ ) of an enzyme reaction. *Biochem. Pharmacol.* 22: 3099-3108, 1973.
20. Hill, A. V. The possible effects of the aggregation of the molecules of hemoglobin on the dissociation curve. *J. Physiol. London* 40: iv-vii, 1910.
21. Ellman, G. L., K. O. Courtney, V. Andres, and R. M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7: 88-95, 1961.
22. Benke, G. M., K. L. Cheever, F. E. Mirer, and S. D. Murphy. Comparative toxicity, anticholinesterase action and metabolism of methylparathion and parathion in sunfish and mice. *Toxicol. Appl. Pharmacol.* 28: 97-109, 1974.
23. Rimele, T. J., W. A. Rogers, and T. S. Gaginella. Characterization of muscarinic cholinergic receptors in the lower esophageal sphincter of the cat: Binding of [<sup>3</sup>H] quinuclidinyl benzilate. *Gastroenterology*, 77: 1225-1234, 1979.
24. Gaginella, T. S., T. J. Rimele, T. M. O'Dorisio, and R. J. Dorff. Pharmacologic identification of muscarinic receptors in the pylorus of the cat by binding of [<sup>3</sup>H] quinuclidinyl benzilate. *Life Sci.* 26: 1599-1608, 1980.
25. Gilbert, R. F. T., M. R. Hanley, and L. L. Iversen. [<sup>3</sup>H]-quinuclidinyl benzilate binding to muscarinic receptors in rat brain: Comparison of results from intact brain slices and homogenates. *Br. J. Pharmacol.* 65: 451-456, 1979.

26. Williams, L. T., and R. J. Lefkowitz. Receptor binding studies in adrenergic pharmacology. Raven Press, New York, 1978.
27. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* 14: 723-736, 1978.
28. Schimerlik, M. I., and R. P. Searles. Ligand interactions with membrane-bound porcine atrial muscarinic receptor(s). *Biochemistry* 19: 3407-3414, 1980.
29. Halvorsen, S. W., and N. M. Nathanson. In vivo regulation of muscarinic acetylcholine receptor number and function in embryonic chick heart. *J. Biol. Chem.* 256: 7941-7948, 1981.



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